



Universidade do Algarve
Faculdade de Ciências e Tecnologia

**OPTIMIZATION OF MANAGEMENT PROCEDURES
FOR SEA URCHIN (*Paracentrotus lividus*)
ARTIFICIAL REPRODUCTION**

Daniel Alexandre Cardoso Rodrigues

Master degree Thesis in Aquaculture and Fisheries

Prof. Doutora Elsa Cabrita

Doutora Florbela Soares

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Abbreviations and acronyms

°C – degrees Celsius

μL – microliter

DNA – deoxyribonucleic acid

fps – frames per second

FSW – filtered sea water

L – liter

M – molar

min – minutes

mL – milliliter

PBS – phosphate buffered saline

SP – subpopulation

SW – sea water

tons – tonnes

Index

Acknowledgements/Agradecimentos	3
Abbreviations and acronyms	4
Index	5
Index of Figures	7
Index of Tables.....	7
Abstract	8
Resumo	10
1. State of the art	12
1.1 Aquaculture industry	12
1.2 Sea urchin harvesting and production.....	12
1.3 Sperm importance	14
1.4 Factors affecting sperm quality	15
1.5 Sperm quality evaluation	17
2. Objectives.....	22
3. Material and Methods	23
3.1 <i>Paracentrotus lividus</i> broodstock	23
3.2 Gamete collection.....	24
3.3 Sperm Analysis.....	25
3.4 Sperm characterization between populations and generations.....	27
3.5 Optimization of fertilization outcomes.....	27
3.6 Preservation techniques	Erro! Marcador não definido.
3.7 Statistical analysis	30
4. Results	31
4.1 Wild population from North vs South of Portugal.....	31
4.2 Wild population (F0), F1 and F2.....	33

4.3	Sperm to Oocyte ratio.....	35
4.4	Gamete contact time	36
4.5	Cryopreservation vs preservation.....	37
5.	Discussion.....	39
5.1	Spermatozoa characterization of diferent populations	39
5.2	Fertility trials	40
5.3	Post-thaw and preservation quality analysis on <i>P. lividus</i>	41
6.	Conclusion	43
7.	References.....	44

Index of Figures

Figure 1 - Fiber glass tanks where sea urchin (<i>Paracentrotus lividus</i>) is maintained at EPPO	23
Figure 2 - Induced spawning technique	24
Figure 3 – Percentage of spermatozoa per subpopulation of four populations of <i>P. lividus</i> at 5 and 10 minutes post-activation.....	32
Figure 4 – <i>P. lividus</i> percentage of fertilization rate in function to the spermatozoa to oocyte ratio.....	35
Figure 5 – Fertilization rate obtained is using different gamete contact time	36

Index of Tables

Table 1 - Mean values and standard deviation of viability and motility from wild South and North <i>P. lividus</i> spermatozoa at 5 min and 10 min post activation	31
Table 2 - Mean values and standard deviation of cell viability and total motile cells data of F0, F1 and F2 generation at 5 min and 10 min post activation.....	33
Table 3 - Mean values and standard deviation of viability, motility and DNA fragmentation data collected from preserved and cryopreserved sperm samples	37

Abstract

Aquaculture industry has a huge contribution for the world food production, and is extremely necessary to develop and optimize the production techniques, especially not to depend totally on wild stocks. In the case of the sea urchin *Paracentrotus lividus*, the demand increase of their roe is provoking a drastically decrease of their wild stocks, particularly in the recent years. This demand for sea urchin roe is arousing interest for the industry for a new profitable product and for the recovering and preserve wild populations. In this sense, new research needs to guarantee optimization of the techniques used to reduce the effort and the production cost. The selection of breeders is a very important aspect for sea urchin industry, since the success of the reproduction and quality of their product are dependent of the breeders. In addition to the high cost of acquiring wild specimens, it is not viable in the long term. Therefore, is important to study the possibility of use breeders from captive generations. The optimization of *in vitro* reproduction techniques is crucial for an efficiency production. The time that gametes need to be in contact for the best fertilization rate and the ratio of spermatozoa per oocyte are two of the principal aspects to be considered in an optimization of an *in vitro* reproduction protocol. Through preservation and cryopreservation technology is possible to promote alternatives techniques to contribute for the resource management efficiency of *P. lividus*. The objectives of this work are to study the hypothesis of using male wild sea urchins from different locations and captive generations specimens as breeders, optimize *in vitro* protocols using different contact times and different spermatozoa to oocyte ratio, and compare cryopreservation and preservation techniques for the storage of the sperm. To study the possibility of use captive generations as breeders were evaluated the sperm quality of each case, for this evaluation were analyzed the viability and motility, being used two approaches for the last one (mean values and subpopulation). The optimization of fertilization outcomes was evaluated counting the number of fertilized eggs using a counting chamber (Sedgwick) in optical microscope. Moreover, the comparation of the preservation and cryopreservation techniques was analyzed the motility by mean values, viability and DNA fragmentation (comet assay). The motility analysis were assessed by CASA system, viability through staining techniques and counting at a fluorescent microscope, DNA

fragmentation was quantified the DNA tail. Significant differences were observed ($p < 0.05$) in breeders selection, revealing the domestication seems to have a positive effect in *P. lividus* sperm quality. The *in vitro* spermatozoa to oocyte ratio trial revealed significant differences between all ratios, where the 20 000 spermatozoa to oocyte ratio had the higher value. Contact times chosen differed negatively from the control. The preservation technique had higher viability in the first 6 days comparing to cryopreservation, although had no damage in DNA were detected in both. The present study revealed that domestication seems to be a good step to optimize the breeders sperm quality. With the higher spermatozoa to oocyte ratio used is possible to reach almost the total egg fertilization percentage. The preservation at 4°C is a good technique to preserve the sperm quality until 6 days, cryopreservation seems to be better from there if the protocol is optimized.

Key words: sperm; *in vitro* reproduction; cryopreservation; breeders selection; *Paracentrotus lividus*

Resumo

A indústria de aquacultura tem uma enorme contribuição para a produção de alimentos a nível mundial, e é extremamente necessário para desenvolver e otimizar as técnicas de produção, especialmente para não depender totalmente nos stocks selvagens. No caso do ouriço do mar *Paracentrotus lividus*, o aumento da procura das suas ovas tem provocado uma descida drástica dos stocks selvagens, particularmente nos últimos anos. Esta procura pelas ovas de ouriço do mar tem despertado o interesse da indústria para a produção de um novo produto lucrativo e para a recuperação e preservação das populações selvagens. Neste sentido, os futuros estudos devem garantir a otimização das técnicas usadas para reduzir o esforço e o custo da produção. A seleção dos progenitores é um aspeto muito importante para a indústria do ouriço do mar, visto que o sucesso da reprodução e a qualidade do produto final é dependente dos mesmos. Além do elevado custo da aquisição de espécimes selvagens, não é viável a longo termo a sua captura no meio natural. Portanto, o estudo da possibilidade de utilizar progenitores descendentes dos reproduzidos em cativeiro é bastante importante. A otimização das técnicas de reprodução *in vitro* é crucial para uma reprodução eficiente. O tempo que os gametas necessitam de estar em contacto para a melhor taxa de fertilização e o rácio de espermatozoides por oócito são dois dos principais aspetos a considerar na otimização de um protocolo de reprodução *in vitro*. Através de tecnologias de criopreservação e preservação é possível utilizar técnicas alternativas que podem contribuir para um manuseio de recursos mais eficiente no caso do *P. lividus*. Os objetivos deste trabalho englobam o estudo da hipótese do uso de espécimes de ouriços do mar macho capturados em diferentes localizações geográficas e gerações de cativeiro como progenitores, otimização de protocolos *in vitro* usando tempos de contato e rácios espermatozoide para oócito diferentes, e comparar técnicas de criopreservação e preservação para o armazenamento do sémen. Para estudar a possibilidade do uso de espécimes selvagens e de diferentes gerações de cativeiro como progenitores foi avaliada a qualidade do sémen de cada caso, nesta avaliação foi analisada a viabilidade e a motilidade dos espermatozoides, tendo sido usadas duas abordagens diferentes para a última (valores médios e subpopulações). A otimização das técnicas de fertilização foi avaliada através da contagem do número de ovos

fertilizados usando uma camera de contagem (Sedwick) num microscópio ótico. Por fim, na comparação das técnicas de criopreservação e preservação foi analisada a motilidade através de valores médios, a viabilidade e a fragmentação do ADN (teste de cometas). A análise da motilidade foi efetuada usando o sistema CASA (Computer-assisted sperm analysis), viabilidade através de técnicas de coloração fluorescente e contagens num microscópio de fluorescência, a fragmentação do ADN avaliada através da quantificação cauda do ADN. Foram observadas diferenças significativas ($p < 0.05$) na seleção dos progenitores, revelando que a domesticação parece ter um efeito positivo na qualidade do sémen de *P. lividus*. Os ensaios *in vitro* com diferentes rácios de espermatozoide por oócito mostraram diferenças significativas em todos os rácios, sendo que o rácio de 20 000 espermatozoides por oócito teve a maior percentagem de fertilização. Os tempos de contacto escolhidos diferenciaram negativamente do controlo. Comparando com a técnica de criopreservação, a técnica de preservação teve maior viabilidade nos primeiros 6 dias, no entanto não foi detetado nenhum dano no ADN em ambas as técnicas. Este trabalho revelou que a domesticação parece ser um bom passo para a otimização da qualidade do sémen dos progenitores de *P. lividus*. Com o maior rácio de espermatozoides por oócito foi possível atingir quase a percentagem total de ovos fertilizados. A preservação a 4°C é uma boa técnica para preservar a qualidade do sémen até aos 6 dias, depois deste tempo, a criopreservação parece ser melhor, contudo o protocolo necessita de ser otimizado.

Palavras-chave: sémen; reprodução *in vitro*; criopreservação; seleção de progenitores; *Paracentrotus lividus*

1. State of the art

1.1 Aquaculture industry

Aquaculture importance has been growing in the past decades with the need of providing other protein sources apart from the one supplied by fisheries. Between 1990 and 2017, fisheries caused the decreasing of 24% of fish stocks within biologically sustainable levels (FAO, 2020). As a complementary source of protein production, global aquaculture industry developed and raised the production 527% from 1990 until 2018. In 2018, the aquaculture production breaks the all-time record, producing 114.5 million tons. In the same year, about 20.5 million people were employed in aquaculture. It became an important activity with huge contribution for the world food production and employment. The aquaculture industry has focused mainly in a specific niche of finfish species, with a 54.3 million tons produced in 2018. Following finfish were mollusks, with 17.7 million tons. In mollusks, bivalves were the mainly produced (9.4 million tons), followed by marine invertebrates with a production of 435.400 tons. Recently, in aquaculture industry, species diversification has been one of the main goals to achieve.

1.2 Sea urchin harvesting and production

The global market demand for sea urchin in 2014 was around 70.000 tons (FAO, 2014). The sea urchin roe is consumed around the world. Japan is the country that leads the consumption, with about 80-90% of the total current global supply. The market in Europe is not large, consuming only around 5% (Stefánsson et al., 2017). France, Spain and Italy are the mainly sea urchin roe consuming countries in Europe. All around the world, with exception of Japan, sea urchin consumption seems to be novel and trendy, reaching the top 10 food trends in 2016 (Nestlé, 2016). Small niche markets exist in various countries with tendency to grow.

Several species of sea urchin are consumed. *Strongylocentrotus spp.* and *Loxechinus albus* are the most produced ones, although, other species can be more valuable due to the higher quality, different flavour and texture (James, 2017; Sun & Chiang, 2015). The production of some of the more valuable species has been investigated in large commercial scale (Lawrence, 2013). It is in the European market,

mainly France, that sea urchin reach the higher prices as better quality are demanded (Stefánsson et al., 2017).

The purple sea urchin, *Paracentrotus lividus*, has high demand due to its high quality roe (Cook et al., 2007). This seafood is known for its delicate and slightly saline flavour, rich and orange colour (Fabbrocini et al., 2014). This sea urchin species have the highest price in European market and it is the favoured species (James, 2017; Stefánsson et al., 2017). In order to preserve and commercialize *P. lividus*, the production of this species has to be efficient, economic viable and sustainable. This production has to cover the demand for seafood and for the restoration of the places where almost population disappeared due to overexploitation of wild resources. The increased demand of *P. lividus* roe led to reduction of wild stocks (Gibson et al., 2016; Domínguez et al., 2015; Addis et al., 2009). This problem is aggravated with the harvest of adult sea urchin moments before spawning, which corresponds to the period where female gonads are completely developed. The harvest at this moment is justified by “roe with better quality” (James, 2017).

However, aquaculture production can contribute to this production sustainability (Ouréns, 2013). All around the world, the aquaculture of sea urchin became an interesting and valuable business (Sun & Chiang, 2015). Sea urchins can be incorporated in an integrated multi-trophic aquaculture (IMTA) as a primary species (Grosso et al., 2020). This interest in sea urchin aquaculture soared from the 2000 year (FAO, 2016). The species produced are dependent on the existing species in the local country (Eddy et al., 2015; Williamson, 2015; Liu & Chang, 2015; James et al., 2015). For a viable production, it is necessary to deepen the knowledge of the sea urchin species biology and improve the techniques of artificial reproduction and larval rearing.

Sea urchins are dioecious, however, no external differences can be detected between male and female (Hannon et al., 2015). The sexes only can be identified at spawning moment, where males release whitish fluid, while females release a yellow/orange colour and more viscous fluid, or by dissection and observation of the gonads. The gametes are released to water column where the fertilization take place. After 2-3 days, a swimming veliger larvae hatch from the fertilized egg. After this planktonic stage and some metamorphosis stages, occurs settlement and post larvae

become benthic (Rahman et al., 2014). Until reach juvenile phase, sea urchins suffer some more metamorphosis, at these stages are sensitive to chemical signals in water column (Azad et al, 2010). Around 2 years after born, depending on the species, sea urchins develop five separated gonads, each gonad is connected to the upper aboral surface by individual gonopores. The gonads have two functions, produce gametes and storage nutrients within gonadal cells known as phagocytes (Walker et al., 2005).

In aquaculture, sea urchin are produced using *in vitro* fertilization using female and male gamete pools (Mendes et al., 2019). For that, gametes extraction can be achieved using different spawning induction techniques, such as thermal, salinity and mechanical shocks, addition of a specific concentration of microalgae, addition of con-specific gametes and injection of 0.5M KCl to the coelomic cavity. This last procedure proved to be the more efficient, reliable and most used worldwide with *P. lividus* and with various sea urchin species (Gago & Luís, 2011). A proper management of germ cells is crucial to optimize fertilization process and to guarantee healthy juveniles. However, there are no studies about sea urchin sperm quality. Whence, is extremely important to deepen the knowledge on this topic to improve reproduction protocols.

1.3 Sperm importance

The research conducted in the reproduction of any aquatic species focus mainly in egg quality as the responsible for normal larval development (Bobe & Labbé, 2009; Lahnsteiner et al., 2009). Only recently sperm has gain more attention specially because of aspects such as male genetic compatibility with the female, undamaged genome and the specific mRNAs present in spermatozoa that are important factors of male gamete quality and can affect fertilization success and embryo development (Cabrita et al., 2009; Miller et al., 2005).

For aquaculture industry purposes, the use of high quality sperm is a requirement to ensure viable and effective artificial fertilization, it is also important to ensure the high quality and valuable offspring. The selection of males with high quality sperm allow to select better breeders, create better traits with less cost and facilitate the sperm storage (Cabrita et al., 2009; Rurangwa et al., 2004). Therefore, the

identification of factors affecting sperm quality and the development of methodologies that allow its characterization is crucial for the study of any reproductive event.

1.4 Factors affecting sperm quality

The biotic and abiotic factors that can affect sperm quality in aquacultures are quite diverse and dependent on a multiple interaction between physiological, genetic and environment factors.

1.4.1 Broodstock conditions

The broodstock provenience is one of the most important broodstock condition, since there are aspects that are impossible to control. Since sea urchins are benthonic, they are more exposed to water pollutants, such as heavy metals. This exposition is known to affect motility of spermatozoa and impaired gonadal development (Rurangwa et al., 1998; Friedmann et al., 1996). The husbandry conditions can influence sperm quality due to genetic differences and different environment conditions where they were adapted. Nutrition can be a bottleneck due to different eating habits according to the local where wild specimens were caught and should be appropriated for each gonadal development phase.

The physico-chemical aspects of water (temperature, oxygen, salinity), photoperiod, density of individuals, type of tanks among others factors, are important requirements for the production of high quality sperm (Cabrita et al., 2009, 2006). Photoperiod and temperature are the most important factors of broodstock management. The manipulation of these parameters can be employed in aquaculture to accelerate or delay gonadal recrudescence. This control is used to predict the spawn or gonadal development at a convenient time of the year (Nash, 1999). In some species, the modification of the breeding season as well as throughout the breeding period can affect sperm quality (Cabrita et al., 2009).

The sperm quality is significant influenced by the age of the broodstock and may affect the success of sperm storage (Vuthiphandchai & Zohar, 1999). This factor varies widely over species, some have higher sperm quality at first year of reproduction, while

others increase the milt quality over the repeat spawn years, and also, some have a peak along lifetime that can be in the first, middle and later years (Rurangwa et al., 2004).

1.4.2 Spawning induction

Many marine species do not spawn readily in captivity and is necessary the use of induction treatments to induce spermiation or to synchronize gamete release of both breeders (Zohar & Mylonas, 2001). This practice is known to be able to decrease semen quality. The consequences vary according to the technique used and species. Artificial inductions can cause positive and negative consequences to milt. With some treatments and species, after induction, the milt has lower concentration of spermatozoa, while with other treatments the sperm production is higher, with increased motility and longer spermiation period (Rurangwa et al., 2004). In sea urchin, the most used spawning induction technique is the injection of 0.5M KCl to coelomic cavity, as mentioned before. The K⁺ ions depolarize a muscle on the outer surface of the alveolar ovary and testis (Vacquier, 2011), inducing the release of both gametes. It is known that this procedure causes some breeder mortality few days after spawning (Gago & Luís, 2011), although, there is no knowledge about the specific damage that KCl can produce in sperm quality.

1.4.3 Sperm handling

There are no previous studies in sperm quality during handling in sea urchin species, although, in fish sperm this is an important aspect to guarantee the viability of further procedures such as artificial fertilization. From induction, handling, until fertilization moment several factors may affect sperm quality. Feces and urine contamination at the moment of ejaculation may activate sperm motility prematurely, decreasing the potential of movement, change the seminal plasma composition and osmolarity (Rurangwa et al., 2004). This handling factor may affect the quality of sperm in sea urchins, but it was not investigated yet.

1.4.4 Effects during storage

Artificial reproduction techniques usually involve sperm storage until collection of oocytes. Sperm can be storage by two different techniques: refrigeration, at 4°C (short-term); and cryopreservation in liquid nitrogen, at -196°C (long-term).

Refrigeration technique is used when the sperm will be used in some hours or days. However, spermatozoa start to undergo a degenerative process, apoptosis and necrosis related events, anaerobic conditions and oxidative processes, sedimentation of cells. A reduction of the previous mentioned sperm deleterious effects can be achieved, but even with specific protocols it is impossible to prevent the decrease of sperm quality with refrigeration techniques (Beirão et al., 2019).

Cryopreservation procedures can cause osmotic shock to spermatozoa, cell disruption due to the formation of ice crystals and cryoprotectants can cause some degree of toxicity (Cabrita et al., 2010). These factors can damage the cell structure and specifically DNA (Fabbrocini et al., 2015; Beirão et al., 2011).

1.5 Sperm quality evaluation

High sperm quality is characterized by the ability of the spermatozoa to reach the egg and the capacity to penetrate the egg envelopes (Cabrita et al., 2009). Moreover, spermatozoa needs to maintain its ability to recognize the oolemma, to fuse with oocyte plasma membrane inducing the activation of egg metabolic pathways and in the final to transmit undamaged genome with genetic compatibility with the egg genome (Simmons, 2005).

For some years, the quality of sperm was determined using eggs, by fertilizing ability. However, this quality assessment had some problems such as the availability of eggs can be a bottleneck or the quality of eggs can interfere in the development (Rurangwa et al., 2004). There has been drive research measuring other parameters/biomarkers of sperm quality that relate to sperm fertilization ability (Cabrita et al., 2011). Parameters of sperm quality so far documented in several species, but mostly in fish, include sperm density, osmolarity and pH of seminal plasma as their chemical composition, spermatocrit, enzymatic activity, motility, cell viability, cell

membrane resistance, morphology and ultrastructure, mitochondria viability and functionality, metabolic activity (ATP), fertilizing capacity, DNA integrity and others depending on the objective of the research or application (Cabrita et al., 2010, 2009; Lahnsteiner et al., 2009; Geffen & Evans, 2000; Billard et al., 1995).

1.5.1 Sperm Motility

Motility is the most studied biomarker of sperm quality assessment in several species due to its correlation to fertility in some species (Rurangwa et al., 2001). For a long time, motility assay only relied in estimation of the percentage of motile spermatozoa and the total duration of movement. The development of objective methods of computerized assisted evaluation (CASA), transformed the evaluation of sperm motility much more accurate and can assess different sperm motility traits such as the type of movement, trajectory and velocity of spermatozoon (Cabrita et al., 2009). These characteristics can be studied through the analysis of the following sperm motion variables: curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), linearity (VSL/VCL), straightness (VSL/VAP), wobble (a measure of oscillation $WOB = VAP/VCL$), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF). The motility data can be analyzed as mean values of all spermatozoon population or using a cluster analysis, where cells are classified according to each individual characteristics. This method follows the principal that an ejaculate is composed by a heterogeneous population of spermatozoa with different characteristics. The cluster analysis allows to detect the presence of subpopulations in sample based on differences in motility parameters of each spermatozoa cell (Beirão et al., 2011). Therefore, this method is one of the most reliable assays to perform in order to analyze sperm quality.

1.5.2 Sperm viability

Cell viability is an assay that measures spermatozoa individual status and is related with plasma membrane integrity. Plasma membrane is a key structure responsible for the reception of stimulus and the triggering of responses, such as activation of motility after dilution in appropriate media or fusion with oocyte plasma membrane. Therefore, alterations at plasma membrane level can modify their

permeability and functionality. For these reasons, the evaluation of plasma membrane integrity and permeability is considered an index of cell viability, since a cell with damage at plasma membrane should be unable to develop its functions (Cabrita et al., 2009). The viability test can be performed by the use of two fluorescent dyes, such as SYBR green and propidium iodide, one permeable to intact membrane and other that only stain cells in case of membrane damage, respectively (Cabrita et al., 2011).

1.5.3 DNA integrity

The DNA integrity evaluation is an important assay to evaluate sperm quality, because spermatozoa can be viable in terms of membrane structure and have motility but may have some DNA fragmentation in the nucleus. The chromatin condensation with proteins and the presence of antioxidants in the seminal plasma protect the DNA from injuries until spermatozoa reach the egg. The degree of chromatin condensation is correlated to the DNA integrity. To evaluate chromatin damage, several methods can be used, being terminal deoxynucleotidyl transferase-nick-end-labelling (TUNEL), the sperm chromatin structure assay (SCSA) and Comet assay (single cell gel electrophoresis -SCGE) the most used. All of them can detect double or simple strand breaks in the DNA double helix. Comet assay is a versatile, sensitive and inexpensive method to assess breaks in individual cells, and has been widely used in sea urchin spermatozoa toxicity research (Oliviero et al., 2018; Cabrita et al., 2009; Collins, 2004).

1.6 Sperm management techniques

There are several ways to manage sperm to perform further artificial fertilization when eggs are not immediacy available or when the genetic pool of a certain individual needs to be stored. Sperm can be just preserved at 4° C (preservation) to maintain viability for several hours, days or even weeks or cryopreserved at very low temperatures theoretically maintaining its viability indefinitely.

The preservation technique is simple and does not required expensive equipment or complex procedures. Yet, as mentioned before, the sample quality

decrease quickly along time, this decreasing quality rate depend on the species and conditions used (Beirão et al., 2019).

Cryopreservation allows to use preserve intact live samples for long-term, this duration depends to the species and the quality of the material prior and after the procedure. Therefore, cryopreservation is a powerful tool for management of a species reproduction in aquaculture. In aquaculture, the sperm can be stored until the eggs are available, the excess sperm can be storage and used when semen is difficult to obtain (for example: low volume of semen stripped, low quality sperm) and facilitates the transport of gametes to different fish farms. It can also be used to store the genome of a valuable strain allowing to maintain the desired fish characteristics (Cabrita et al., 2010). Thus, sperm cryobanks are undoubted useful for farms as for conservation programs to create gene banks (Labbé et al., 2013; Hiemstra et al., 2006).

1.6.1 Cryopreservation

Cryopreservation is a process that implies the decrease of temperature to preserve the biological material through a specific rate until -196°C . Once the sample is frozen, it allows the storage at mid and long term (Day et al., 2018). Biochemical reactions and all biological processes are interrupted, maintaining the biological material intact until thawing, moment of when the cells recover their metabolic activity (Cabrita et al., 2011). Although, this process can cause damage at cellular level, the use of certain substance, named cryoprotectants, added to the cell media reduce this damage and allow the quality maintenance of the cryopreserved sample. Apart from that, their addition reduces the freezing injury by stabilization of intracellular proteins, reducing or eliminating lethal intra- and extracellular ice crystal formation, and minimize the impact of concentrated intra- and extracellular electrolytes (Elder & Dale, 2020). Dimethyl sulfoxide (DMSO) is, by far, the most widely used cryoprotectant for sperm in aquatic species (Labbé et al., 2013). In sea urchin, DMSO provided a good protection at concentrations of 5-20% (Fabbrocini et al., 2014; Gwo, 2000).

The freezing and thawing velocity rates are also crucial to the survival and to the recovery of cell metabolism without cellular damage.

During cryopreservation cells need to resist to several processes. Freezing causes cell surrounding solution to concentrate, consequently the cells shrink due to dehydration caused by cryoprotectants hyperosmotic medium. The slower it is the freezing rate, more time the sample will be exposed to a hyperosmotic medium and the risk of dehydrate is higher. However, if the freezing rate is too fast, intracellular crystallization can lead to cell damage (Pegg, 2007). Therefore, an equilibrium needs to be reach and adapted for each species.

Thawing is a process that allows cells to recover their metabolism and activity by warming the sample. Usually, is performed by immersing the sample container in a water bath or in air at ambient temperature. This procedure should be as fast as possible to avoid the reorganization of the intra and extracellular ice crystals (Elder & Dale, 2020).

There are different equipment to freeze samples. Simplified systems can be used, such as a dry ice bath, a rack set at different heights above or floating on a liquid nitrogen surface. However, controlled rate freezer devices are often used and have absolute control of cooling temperatures, although, these equipment are often expensive (Paredes et al., 2019). Typically, to store samples for cryopreservation, plastic straws of 0.25 or 0.5 mL or cryovials are used (Martínez-Pastor & Adams, 2008).

2. Objectives

The general objective of our work was the optimization of management procedures for sea urchin (*Paracentrotus lividus*) *in vitro* reproduction. To achieve this objective some specific objectives were defined: study the sperm quality of wild sea urchins from two different locations and two captive generations (F1 and F2); optimize the reproduction protocol by analyzing different spermatozoa to egg ratios and the gamete contact time necessary to occur fertilization; evaluate two sperm storage methods (preservation and cryopreservation).

3. Material and Methods

3.1 *Paracentrotus lividus* broodstock

Wild *Paracentrotus lividus* were captured in South and North coast of Portugal in summer of 2016 and spring of 2020, respectively. Then, individuals were transported to IPMA facilities in Olhão (EPPO). The wild sea urchins (males and females) from South coast (WS) and North coast (WN) were maintained, separately, in 8 000L fiberglass tanks. The first captive generation (F1) was born in the summer of 2017, the breeders were the WS sea urchins (F0). F1 sea urchins were maintained in 300L fiberglass tanks, Figure 1. The second captive generation (F2) was born in the summer of 2018, the breeders were the F1 sea urchins. This generation was maintained in 110L fiberglass tanks. The tanks conditions were the more natural possible. Individuals were maintained in natural photoperiod (14L:10D during the breeding season), with natural water temperature (the temperature in the month before and during the essays was 21-22°C) and salinity (36‰). All sea urchins were fed with sea lettuce (*Ulva sp.*) and corn (*Zea mays*). The tanks had an open water system with a flow rate of 7L/min for the tanks with wild specimens, 4.5 and 16L/min for tanks with F1 and F2 sea urchins, respectively. The different tanks flowing rates were due to the different tank sizes, allowing in all cases a correct renewal of all water. A total of 10 (WN), 10 (WS), 10 (F1) and 27 (F2) males were used. The total number of *P. lividus* females utilized was 6 from the second generation.

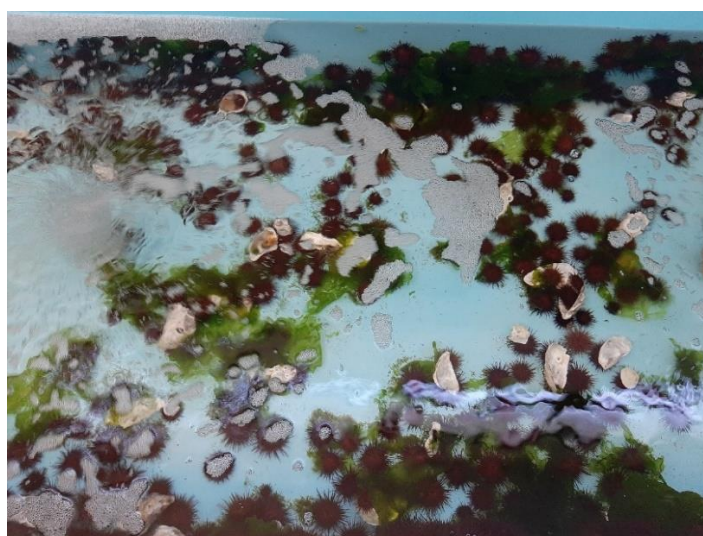


Figure 1 - Fiber glass tanks with 300L capacity where F1 sea urchin (*Paracentrotus lividus*) generation is maintained at EPPO, Estação Piloto de Piscicultura de Olhão (Instituto Português do Mar e da Atmosfera facilities in Olhão, Portugal)

3.2 Gamete collection

For each experiment, individuals were injected into the coelomic cavity with 1mL of 0.5M KCl, Figure 2 (b), and then placed inverted on top of a beaker to carry out gamete collection (Mendes et al., 2019). The female and male, Figure 2 (c) and (d) respectively, sea urchins that released gametes were separated.

The sperm was collected, as they were extruded from the gonopore, with a micropipette to a 1 microtube and stored in a box with ice, Figure 2 (a). The oocytes released were washed with sea water (SW) to a beaker and stored in a box with ice. The gametes that were visible contaminated with feces were discarded, like those with strange texture (for example: very liquid, strange colour).

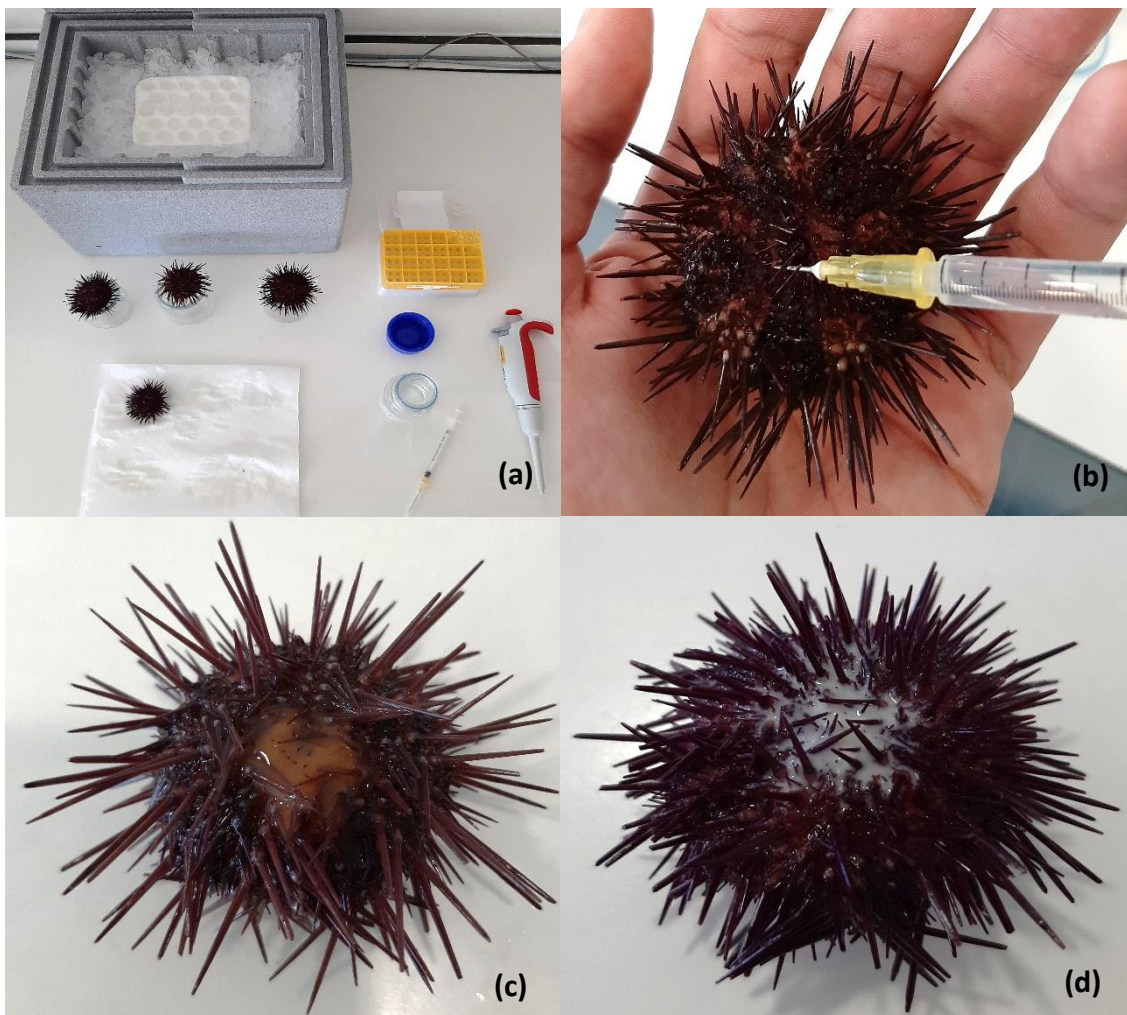


Figure 2 - Induced spawning technique. (a) Material used to perform artificial spawning; (b) Injection of potassium chloride in *Paracentrotus lividus*; (c) Release of female gametes, orange color; (d) Release of male gametes, whitish color.

3.3 Sperm Analysis

Three sperm quality analysis were performed namely: motility, viability and comet assay.

3.3.1 Motility

Spermatozoa motility was performed on fresh and post thawed samples. The sperm samples were diluted/activated 1:100 with filtered sea water (FSW) (1 μ L of sperm to 99 μ L FSW), except the post thawed samples. These samples were diluted 1:10, since they were cryopreserved with a 1:10 dilution.

For the assessment of total motile cells (TM) and motility parameters (VCL, VSL, VAP, LIN STR, WOB, ALH and BCF) Computer Assisted Sperm Analysis (CASA) software (ISAS – Integrated Semen Analysis System, Proiser R+D, S. L., Spain) was used. The equipment used was a Makler chamber introduced in a phase-contrast microscope (Nikon E200, Japan) with a 10x negative objective, a digital camera (Basler A312f C-mount, Germany) set at 25 fps. The settings for CASA were: 1 < Particles Area (in microns²) < 90; VCL: 10 < slow < 45 < medium < 100 < rapids (microns/sec); progressivity: 80% of the STR; connectivity: 12; minimum number images to calculate ALH: 10. These settings were previously set for other species (Beirão et al., 2009) and adapted for sea urchin sperm.

3.3.2 Viability

Spermatozoa viability was performed on fresh and post thawed samples. The sperm samples were diluted 1:100 with PBS (1 μ L of sperm to 99 μ L PBS), except the post thawed samples. These samples were diluted 1:10, once sperm was cryopreserved with a 1:10 dilution.

For this analysis, 0,5 μ L of SYBR green (1 000x concentrated in DMSO) (SYBR 14 - Sigma, Portugal) and 1 μ L of 2.4mM propidium iodide (PI - Sigma, Portugal) were added to 100 μ L sperm diluted sample. After 3 minutes, 6 μ L of sample were dropped in a slide with coverslip and placed in the fluorescent microscope (Nikon Eclipse E200) for more 3 minutes. Subsequently, the samples were observed and images were recorded with a digital camera (VisionCam5, WWR) and the imaging software NIS Elements v3.01 (Nikon,

Spain). Three photos with at least 100 cells each were taken. Afterwards, the number of live cells (stained with green dye SYBR-14), and dead cells (cells permeable to the PI dye stained with red) were counted.

3.3.3 Comet assay

For DNA fragmentation test (Comet assay or single cell electrophoresis assay), the protocol described by Cabrita et al., (2005) was used with some adaptations for sea urchin sperm. The slides were covered with 60 μ L agarose (normal agarose 0,5% in PBS) with a smear. When the agarose in slides was completely dried, the sperm was diluted 1:1000 with 1% NaCl solution and, 20 μ L of diluted sperm was added to 150 μ L of low melting point agarose (0.5% in PBS). For each slide 60 μ L of the previous solution was pipetted two times and covered with two coverslips, then placed horizontally at 4°C to solidify. After 15 minutes, the coverslips were taken out with a gentle and quick movement. The samples were exposed to a lysis solution (2.5M NaCl, 100mM Na₂-EDTA, 10mM Tris (base) pH10, 1% Triton X-100, 1% lauryl sarcosine) for 1 hour at 4°C. After lysis, the slides were placed horizontally in an electrophoresis cube (BioRad, Spain) filled with electrophoresis solution (0.3M NaOH, 1mM Na₂-EDTA, pH 13). After 20 minutes of unwind, alkaline electrophoresis was conducted for 20 minutes at 25V and 300mA at 4°C. After it, the slides were removed and neutralized (0.4M Tris-HCl, pH 7.5) with two washes of 5 minutes at 4°C and then fixed in 98% ethanol for 3 minutes.

For comet visualization, 10 μ L of 0.24 μ M propidium iodide were pipetted into the sample, covered with a coverslip. After 5 minutes, slides were placed in the fluorescent microscope and photographs taken of at least 100 comets for each slide (the equipment and software used was the same as used with viability). The comet analysis was performed with the imaging system Komet software v6.0 (Andor Technology, Ireland). DNA fragmentation was recorded as percentage of DNA in tail (%DNA_t).

3.4 Sperm characterization between populations and generations

3.4.1 Wild populations from North vs South of Portugal

The viability and motility tests were performed on sperm samples from two different wild populations of sea urchin, North (WN) and South (WS) coast of Portugal adapted to captivity. For this analysis, sperm samples were used from 10 males from each location. The protocols followed for viability and motility tests were the same that were described before in "Sperm Analysis". Using the same sample, the motility data was collected 5 minutes post-activation and 10 minutes post-activation with seawater.

3.4.2 Wild population (F0), F1 and F2

The same analysis performed previously were made between wild population (F0), first generation offspring (F1) and second generation offspring (F2).

3.5 Optimization of fertilization outcomes

In order to improve the fertilization protocol two different assays were performed. The first one tried to understand what was the best sperm to egg ratio. The second one tried to optimize gamete contact time during fertilization.

3.5.1 Sperm to egg ratio

The gametes, sperm and oocytes, used to this essay were from six F2 sea urchins, three males and three females. Four different sperm to oocyte ratios: 100:1, 500:1, 5 000:1 and 20 000:1 were tested. Collection of gametes was performed as described before.

The number of oocytes was determined in a Sedgewick-Rafter cell-counting chamber in an optical microscope (Nikon Eclipse E200) and concentration per milliliter was calculated. Then, oocytes were mixed in a pool to use the same oocyte quality in all fertilizations and distributed by the 36 fertilization beakers (3 males x 4 sperm:egg ratio x 3 replicates), 10 000 oocytes per beaker. Each beaker had 50mL of filtered seawater.

For the calculation of sperm concentration, CASA software was used as described previously. After quantifying sperm concentration, the dilutions were calculated, to each male, to accomplish 10×10^8 spermatozoa/mL. For each fertilization, the necessary amounts of activated sperm from each male were pipetted into the beakers containing eggs. Due to the high precipitation of oocytes, the beakers containing the gametes were gently mixed every 30min during 1.5 seconds. After fertilization (120 minutes), eggs were centrifuged at 500g for 5min to eliminate seawater, fixing the eggs with 4% PFA for 10min, washed twice with distilled water, using the same centrifugation process, and stored in distilled water.

From each beaker, the number of fertilized and no fertilized eggs were counted in triplicate with a counting chamber (Sedgwick-Rafter) in optical microscope.

3.5.2 Gamete contact time

The gametes, sperm and oocytes, used to this essay were from seven F2 sea urchins, four males and three females. Three different contact times: 3min, 10min and 120min were tested. The sperm to oocyte ratio used was 20 000 spermatozoa/oocyte. Collection of gametes were performed like described before.

The determination of gamete concentration and dilution followed the same protocol described previously.

A pool was made with oocytes from the different females, then distributed by 36 fertilization beakers (4 males x 3 contact times x 3 replicates), 10 000 oocytes per beaker. Then, at the same time sperm was pipetted at the necessary amounts from each male to the beakers. At 3min, 10min and 120min, 10mL 0.55M KCl (prepared in sea water) were added to the corresponding vials to stop sperm motility and consequent fertilization process. Oocyte preparation for fertilization determination was done as previously described.

Three replicates from each sample were taken for counting. The number of fertilized and non-fertilized eggs were counted with a counting chamber in an optical microscope.

3.6 Storage Methods

Two preservation techniques were tested and sperm quality evaluated according to the protocols previously described.

3.6.1 Cryopreservation

The cryopreservation trial was performed with sperm from five F2 males. The cryoprotectant used was DMSO at a 10 % concentration and the medium was FSW (filtered sea water). Sperm was diluted 1 to 10 in cryopreservation medium, being loaded into 500 μ L straws. A programmed biofreezer (Asymptote EF600 – Grant, England) was used to perform a -7 $^{\circ}$ C/minute freezing rate until reach -70 $^{\circ}$ C (Paredes et al., 2019), and then samples were plunged directly into liquid nitrogen.

Samples were preserved for a month. Following the previous mentioned protocol, thawing was performed in a water bath at 30 $^{\circ}$ C for 15 seconds. Samples were immediately used to evaluate sperm quality, through motility and viability tests (as described before) and DNA damage (Riesco et al., 2017).

3.6.2 Preservation at 4° C

Sperm samples from five F2 males were extracted and stored at 4 $^{\circ}$ C for 10 days. The sperm quality was evaluated, through motility and viability tests (as described before) and DNA damage (only in the day 0). The sperm quality evaluation was performed in the first day (day 0), and then every two days (day 2, 4, 6, 8 and 10). The final day was defined as the day when most of the samples lose completely the mobility.

3.7 Statistical analysis

Motility data were extracted, as means values, from software CASA to excel format, with exception of data to be used in subpopulations analysis. In the last case, the individual spermatozoa data was extracted.

To perform subpopulation analysis the software RStudio (TM v.1.3.1073.0) was used. Data in excel format was imported to this software. Then, a k-means cluster analysis was performed, generating clusters that characterized different sperm populations. All spermatozoon was labelled with a number corresponding to each subpopulation. Data with this label was exported in excel format.

To carry out statistical analysis of motility, viability, subpopulations and fertilization data the software SPSS (IBM SPSS Statistics v.22) was used. In all data normality was checked. Three different situations were detected: data with normal distribution; data without normality that was transformed using $\arcsin(\sqrt{x})$ to have normal distribution; data that did not get normality after transformation. In the first and second situation a one-way or two-way ANOVA was used, depending on the pretended comparison, with SNK as post-hoc test. In the last situation, the non-parametric test Kruskal Wallis was applied.

4. Results

4.1 Wild population from North vs South of Portugal

Viability

The mean values of sperm viability from North and South sea urchin wild populations are presented in table 1. Statistical analysis demonstrated that there were no significant differences in cell viability between populations.

Table 1 - Mean values and standard deviation (n=20) of cell viability and total motile cells (%) collected from sperm samples of wild South (WS) and wild North (WN) sea urchin (*Paracentrotus lividus*) at 5 min and 10 min post activation. For viability data, a Kruskal Wallis test was applied and for total motility a two-way ANOVA (SNK) was used. Significant differences are signed by different letters (p<0.05).

		WS	WN
Cell viability		97.5 ^a ± 0.9	96.1 ^a ± 4.6
Total Motility	5 min	15.1 ^a ± 11.5	6.8 ^b ± 6.0
	10 min	3.8 ^c ± 3.9	0.8 ^c ± 0.6

Motility

The sperm TM (total motility) results from the two wild populations showed significant differences at 5 min post activation, Table 1. Population from South had more motile spermatozoa, 15.1%, than the one from North, with only 6.8%.

At 10 minutes post-activation, there were no significant differences in these two populations. South population had 3.8% motile spermatozoa and North had 0.8%. Motility decreased significantly with time post-activation.

Subpopulations

The subpopulation analysis was performed with clusters analysis which required the use of all motile spermatozoa (n = 883), discarding the motile spermatozoa that had less than 20 frames by CASA.

The application of cluster analysis identified 2 subpopulations (SP1 and SP2) at 5 minutes post-activation for WN and 3 (SP1, SP2 and SP3) for WS. At 10 minutes post-activation, 2 subpopulations (SP1 and SP2) were detected in WN, while in WS were detected 3 (SP1, SP2 and SP3). In these subpopulations the mean values of motility parameters are presented in **Appendix 1**. The percentages of spermatozoa per subpopulation are represented in Figure 3.

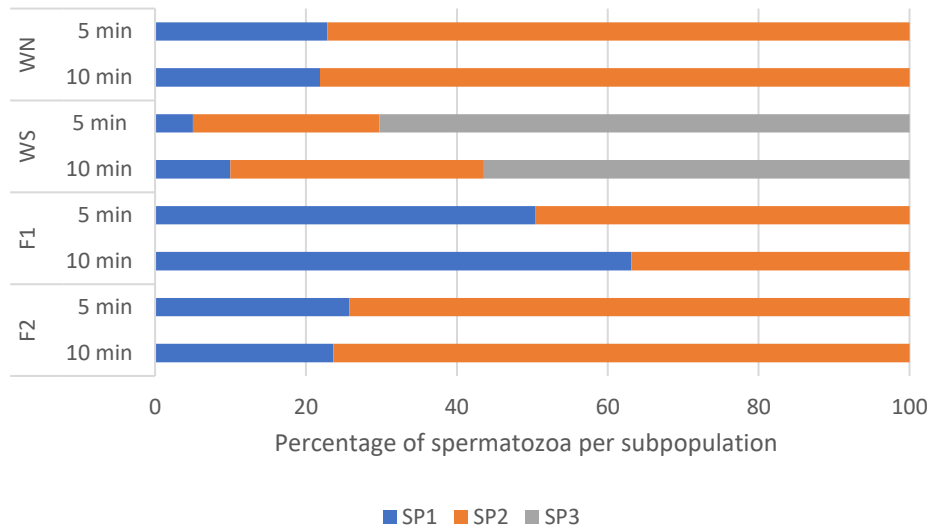


Figure 3 – Percentage of spermatozoa per subpopulation of two wild populations from different locations and two captive generations of *Paracentrotus lividus* at 5 and 10 minutes post-activation.

In WN, at 5 minutes post-activation, 22.84% of motile spermatozoa were grouped in SP1 and 77.16% to SP2. Subpopulation 2 showed low VCL as well low LIN, therefore was considered to be a slow and non-linear subpopulation. SP1 presented high VCL and low LIN, and was considered a fast and non-linear subpopulation. At 10 minutes post-activation, SP1 represented 21.87% of spermatozoa and SP2 78.13%. SP1 was characterized by high VCL and low LIN and, was considered as fast and non-linear subpopulation. SP2 had low VCL and low LIN and was considered to be a slow and non-linear subpopulation. In short, WN was characterized by two subpopulations in both post-activation times, one fast and other slow, both were non-linear. The two subpopulations did not change in proportions along time. The fast subpopulation decreased the velocity along time, as well as the slow subpopulation.

In WS, at 5 minutes post-activation, SP1 had 5% of total cells, while SP2 had 24.77% and SP3 70.23%. SP1 had very high VCL and low LIN, representing super-fast with non-linear trajectory spermatozoa. SP2 grouped together fast and non-linear spermatozoa with high VCL and low LIN. SP3 was characterized by low VCL as well low LIN, being a slow and non-linear subpopulation. At 10 minutes post-activation, SP1 represented 10% of spermatozoa, SP2 33.57% and SP3 56.43%. SP1 was characterized by a very high VCL and low LIN, considered as a super-fast and non-linear subpopulation. SP2 had high VCL and low LIN, being a fast and non-linear subpopulation. SP3 showed low values of VCL and LIN, grouping the slow and non-linear spermatozoa. Summarizing, in WS sea urchins, spermatozoa was distinguished by three subpopulations in both post-activation times, one super-fast, one fast and other slow, all of them were with non-linear trajectories. The proportion of cells per subpopulation changed with time, the number of slow cells decreased and the percentage of super-fast and fast cells increased. The super-fast subpopulation decreased slightly the velocity along time. The fast subpopulation increased, slightly, the velocity.

4.2 Wild population (F0), F1 and F2

Viability

The spermatozoa viability changed between generations. F0 and F1 generations had identical viability values, table 2. However, F2 generation was significant higher with a percentage value of $98.6 \pm 0.9\%$ of live cells.

Table 2 - In this table are represented the mean values and standard deviation (n=30) of cell viability and total motile cells (%) collected from *Paracentrotus lividus* sperm samples of F0, F1 and F2 generation at 5 min and 10 min post activation. For viability data a Kruskal Wallis test was applied and for total motility a two-way ANOVA (SNK) was used. Significant differences are signed by different letters (p<0.05).

		F0	F1	F2
Cell Viability		97.5 ^b ± 0.9	96.1 ^b ± 2.9	98.6 ^a ± 0.9
Total Motility	5 min	15.1 ^c ± 11.5	33.2 ^b ± 17.1	7.5 ^c ± 4.3
	10 min	3.8 ^d ± 3.9	40.4 ^a ± 16.2	1.9 ^d ± 1.6

Motility

The spermatozoa from the three generations (F0, F1 and F2) revealed some TM differences between them. The percentage of motile spermatozoa for each post activation time (5 and 10min), and each generation is present in Table 2. At 5min, F1 generation revealed the higher value, showing 33.2% of motile cells with significant differences regarding other generations. F0 and F2 generation did not show significance differences. At 10min, the results followed the same pattern. The first generation in captive had the higher percentage of motile cells (40.4%), with a significant increase from 5 min post-activation. Other generations had significant decrease along time.

Subpopulations

The subpopulation analysis was performed with clusters analysis which required the use of motile spermatozoa (n = 2061), discarding the motile spermatozoa that was detected with less than 20 frames by software CASA.

The application of cluster analysis identified 3 subpopulations (SP1, SP2 and SP3) at 5 min and 10 min post-activation for F0 and 2 (SP1 and SP2) for F1 and F2. In all subpopulations the mean values of motility parameters are presented in **Appendix 1**. The percentages of spermatozoa per subpopulation are represented in Figure 3.

The F0 results are the same as the result for WS, presented previously (3.Results/3.1.Wild population from North vs South of Portugal/Subpopulations).

In F1, at 5min post-activation, 50.35% of motile spermatozoa were grouped in SP1 and 49.65% in SP2. SP2 presented high VCL and low LIN, and was considered a fast and non-linear subpopulation. Subpopulation 1 revealed low VCL as well low LIN, therefore this subpopulation was considered to be a slow and non-linear subpopulation.

At 10min post-activation, SP1 was represent by 63.13% and SP2 by 36.87%. SP1 was characterized by high VCL and low LIN, being a fast and non-linear subpopulation. SP2 grouped spermatozoa with low VCL and low LIN, and was considered slow and non-linear. In summary, two subpopulations of spermatozoa were detected in F1 sea urchin sperm in both post-activation times, being one fast and the other slow, both with non-

linear trajectories. The proportion of fast spermatozoa increased at 10min. Both subpopulations decreased slightly the velocity along time.

In sperm from F2 males, at 5min post-activation, SP1 was represented by 25.77% of spermatozoa and SP2 by 74.23%. SP1 had high VCL and low LIN, being a fast and non-linear subpopulation. SP2 had low VCL as well low LIN, this subpopulation was considered to be a slow and non-linear. At 10 min post-activation, SP1 had 23.64% of spermatozoa and SP2 had 76.36%. SP2 revealed high VCL and low LIN, and was classified as a fast and non-linear subpopulation. SP1 showed low VCL and low LIN, and was considered to be a slow and non-linear subpopulation. Summarizing, F2 sea urchins revealed 2 subpopulations in both post-activation times, one fast and another slow, being both non-linear. The proportion of motile spermatozoa by subpopulations did not change with time. The velocity and the linearity were the same at both post-activation times.

4.3 Sperm to Oocyte ratio

The result of the four sperm to oocyte ratios are represented in Figure 44.

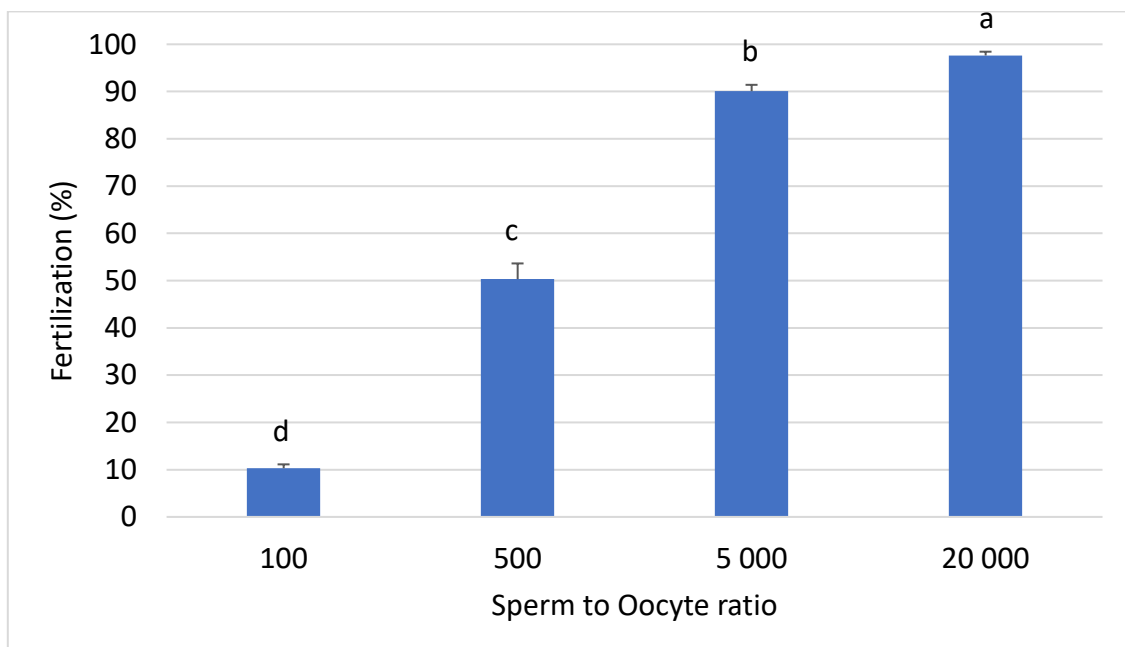


Figure 4 – Fertilization rate (n=36) obtained in *Paracentrotus lividus* as a function of the sperm to oocyte ratios tested (100:1, 500:1, 5 000:1, 20 000:1). A one-way ANOVA was used to compare differences between treatments followed by a SNK test. Significant differences are signed by different letters ($p < 0.05$).

These results showed a higher fertilization rate with higher spermatozoa to egg ratio, 20 000 spermatozoa per oocyte, when compared with other proportions. This ratio, 20 000, had mean fertilization percentage of $97.6 \pm 0.8\%$. The other ratios tested (100, 500, 5 000 spermatozoa per oocyte) were significant different, increasing the fertilization percentage with the increase of number of spermatozoa per oocyte.

4.4 Gamete contact time

The results of the gamete contact time essay are described in Figure 55.

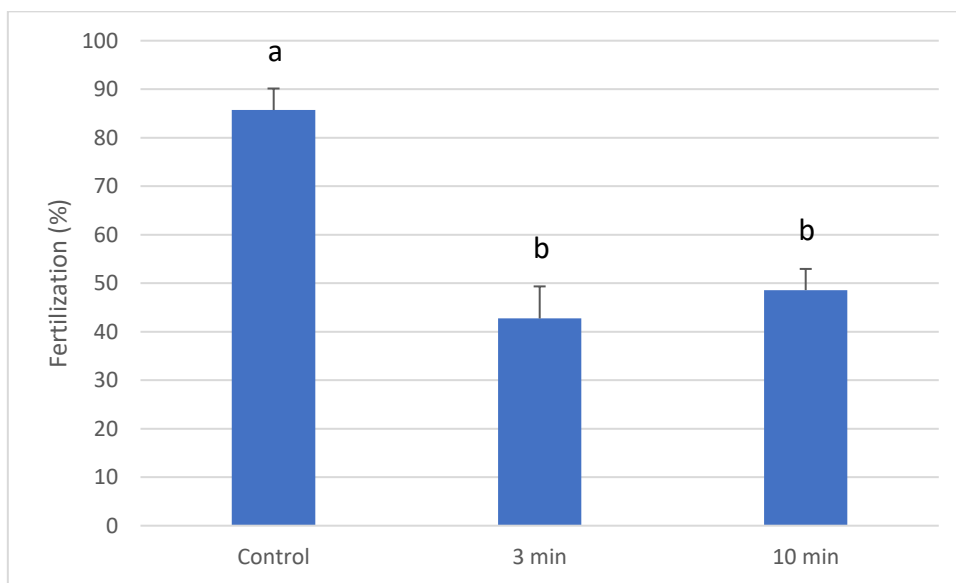


Figure 5 – Fertilization rate (n=36) obtained in sea urchin (*Paracentrotus lividus*) using different gamete contact time, two treatments with addition of KCl at 3 min and 10 min, control with no addition of KCl (120 min). A one-way ANOVA and SNK test were applied to identify significant differences between treatments). Significant differences are signed by different letters ($p < 0.05$).

The addition of KCl at 3 and 10 min to stop fertilization resulted in a fertilization percentage of 42.7 ± 6.6 and $48.6 \pm 4.4\%$ respectively. Both times did not reveal significant differences. However, 3 and 10 minutes were significant different from control samples (no motility arrestment) at 120 min. Control values had an $85.7 \pm 4.4\%$ fertilization rate.

4.5 Cryopreservation vs preservation

The viability values from both preservation essays are presented in Table 3. The statistic test showed two equal subgroups. One included day 0, 2, 4 and 6 and had the higher percentage of viable cells. The other group evidenced that sperm preserved from day 8 to 10 had similar results to the cryopreservation technique used, showing a significant decrease in cell viability.

Table 3 - Mean values and standard deviation of cell viability, motility data collected from sperm samples of *Paracentrotus lividus* preserved during 10 days and cryopreserved during 1 month. DNA fragmentation of fresh (day 0) and post tawed semen. Significant differences were detected by Kruskal Wallis test and are signed by different letters ($p < 0.05$).

Sperm Preservation						
Days	0	2	4	6	8	10
Cell Viability (%)	93.4 ^a ± 2.6	97.3 ^a ± 2.5	97.7 ^a ± 2.7	94.3 ^a ± 2.8	61.92 ^b ± 12.66	62.8 ^b ± 6.8
Total Motility (%)	17.2 ^a ± 8.8	16.0 ^a ± 10.2	22.4 ^a ± 18.9	11.2 ^{ab} ± 11.0	5.2 ^{ab} ± 5.2	1.4 ^b ± 2.6
DNA tail (%)	27.35 ^a ± 15.8					
Sperm Cryopreservation						
Cell Viability	75.4 ^b ± 8.0					
Total Motility	N/A					
DNA tail	27.98 ^a ± 17.10					

The motility analysis in cryopreserved samples showed very low values, near zero. Therefore, these samples were not analyzed. The results from the preservation technique on motility evaluation are present in table 3. The first 8 days did not show any significant difference. Although, the last day showed significant differences between the

first 4 days. The mean value had an ascend pattern until day 4, 22.4% motile cells, and then a descend pattern until the end of the essay. Reaching 1.4% motile spermatozoa.

The DNA damage evaluation revealed that there were no significant differences between fresh and cryopreserved samples. The percentage of DNA in tail in fresh samples was 27.35 ± 15.80 and 27.98 ± 17.10 in cryopreserved samples.

5. Discussion

5.1 Spermatozoa characterization of wild populations and two captive generations

In order to evaluate the quality of the sperm in sea urchin, *Paracentrotus lividus*, were analyzed for the cell viability, total motility and the characterization of spermatozoa subpopulations was performed. Comparing both wild populations and captive generations, the captive generations seem to have higher sperm quality. This fact is clearly demonstrated by motility and viability data since F1 and F2 generations revealed the highest values in motility (33.2% motile spermatozoa and increasing to 40.4% at 10 minutes) and viability (98.6% viable cells). Our results are in opposition with the results obtained by others authors for some fish species where the quality and viability of sperm decreased along captive generations (Sullivan et al., 2020; Valcarce & Robles, 2016). This can suggest that captive generations are more adapted to husbandry conditions (systems, water parameters, handling, daily routines, etc.), meaning in this case that domestication may provoke positive effects in male gamete quality. In wild populations, sea urchins from South coast had higher motility than *Paracentrotus lividus* from North coast at 5 min post-activation. In wild populations, sea urchins from South coast of Portugal had higher motility than *Paracentrotus lividus* from North coast at 5 minutes post-activation. This could be due to the fact that the individuals used from the South coast have been in captivity for a longer period (since 2016) than the North population (2020) that was less adapted to captivity.

In this study, motility cluster analysis was performed in order to evaluate the presence of spermatozoa subpopulations with different characteristics. The parameters rendered for sperm velocity, VCL and VSL, were considered, since these parameters are linked to active sperm motility (Gallego et al., 2015). These two parameters revealed a positive correlation with fertilization capacity in the sea urchin *A. crassispina* (Au et al., 2002). The linearity coefficient (LIN) parameter was used to compare the linearity of subpopulations, because sea urchin spermatozoa have a circular trajectory as normal pattern (Pietschmann, 2009). In this study cluster analysis was performed with motility values of *P. lividus* spermatozoa in order to evaluate the presence of subpopulations

with different characteristics. Despite the more complex and more laborious work than the total motile spermatozoa technique, this approach may constitute a more effective analysis method since sperm is composed by a heterogeneous population of cells with different maturation stages. Sperm subpopulations has been neglected in most fish sperm studies, although, in some species this approach was important to evaluate sperm quality (Beirão et al., 2009; Martínez-Pastor et al., 2006). In our study, the analysis of sperm revealed that motile spermatozoa from wild population from North coast, F1 and F2 generations presented two subpopulations, and that wild population from South coast had three. The parameter that distinguished the subpopulations was the velocity, classified as super-fast, fast and slow subpopulations, all with a non-linear path. Along time, the proportion of cells per subpopulation was the same in WN and F2 but in WS and F1, the slow subpopulations decreased the size, increasing the proportion of cells in fast and super-fast spermatozoa subpopulations. The begin movement of sea urchin spermatozoa is not instantly. This was observed when the pre-trials were performed and is mentioned by some authors that the motility of sea urchin spermatozoa increase and/or stay without alteration some minutes after the beginning of activation (Fabbrocini & Adamo, 2017; Lawrence, 2013). The time necessary to reach the best motility parameters can be related to the time that spermatozoa need to do a final maturation. This aspect has been seen in testicular sperm of several fish and invertebrate species such as catfish, rainbow trout and oyster where spermatozoa need to gain maturation in specific solutions to acquire or even improve motility (Robles et al., 2003).

5.2 Fertility trials

5.2.1 Sperm to oocyte ratio

Fertilization trials were performed to understand what would be the best ratio of spermatozoa per oocyte since information in literature is quite variable. In some cases, the sperm is used as volume and cell concentration or the number of spermatozoa used is not quantified (Brown and Eddy, 2015), inducing concentration errors since the number of spermatozoa can vary between individuals (Smith et al., 2019; Levitan et al., 1991). Different proportions of sperm to oocyte ratio has been used by different

authors, ranging from 500 (according to Mendes et al. (2019) this ratio can be used with good results) to 15 000 spermatozoa per oocyte used by Fabbrocini and Adamo, (2017) as the best ratio for *Paracentrotus lividus* fertilizations. The result from our study revealed that 20 000 spermatozoa was the best ratio to 1 oocyte, obtaining significant lower fertilization results when other proportions were used. The differences obtained between our study and the ones from the other authors could be associated with contact time or even to different motility rates which as demonstrated to influence fertilization successes.

5.2.2 Contact time

The contact time essay was performed to understand how much time is necessary to occur fertilization in this species since spermatozoa in sea urchin can move for longer periods. Contact time is positively correlated to sperm dilution (Levitan et al., 1991). According to Levitan (1991), the fertilization in *Strongylocentrotus franciscanus*, a similar sea urchin species, occurs in the first minutes. Although, our study showed that there were no significant differences at 3 and 10 min gamete contact time, it seems clear that longer periods of time, such as 120min, allowed for a better fertilization rate. The contact time necessary can be changed due to several factors, for example sperm quality level. Viability and motility are negatively correlated to the necessary amount of time to occur fertilization. Low quality sperm need more time to encounter oocytes (Santana et al, 2020), therefore, in sea urchin it seems beneficial to increase gamete contact time to further than 10 min. In further studies it will be necessary to do a timeline from 10 to 120 min to see if higher fertilization rates can be maintained with lower contact time.

5.3 Post-thaw and preservation quality analysis on *P. lividus*

A successful preservation and cryopreservation technique should ensure the maintenance and recovery of the physiological and morphological characteristics of fresh spermatozoa (Beirão et al., 2019, Fabbrocini et al., 2014). In general, the sperm quality from preservation and cryopreservation techniques had differences until day 6 of preservation. One of the easiest methods to test the success of a designed preservation and cryopreservation protocol is performing viability analysis of the

biological material, through membrane integrity and permeability tests (Cabrita et al., 2010). The viability evaluation in preservation and post-thaw samples was done by microscopy using fluorescent dyes and counting each cell individuality to achieve a global percentage of viable cells. The results demonstrated that preserved spermatozoa showed high viability (>90%) and without significant differences in the first 6 days. In the last 4 days (day 8 and day 10) viability decrease and had similar value when compared to cryopreserved ones (60 to 75%). This can be due to the fact that cells undergo a degradation process during preservation because cell metabolism is not stopped only reduced and usually bacteria under these conditions starts to develop. Contrarily, cryopreservation rests cell metabolism but induces cell injuries during freezing/thawing (Cabrita et al, 2008; Beirão et al., 2019). The post-thawed samples had a strange movement, they were vibrating without displacement, and therefore we considered not to evaluate the motility. In preserved samples, motility showed the same tendency as in viability, maintaining the percentage of motile cells in the first 4 days. The constant value in the first days and the final decreasing can be explained by consumption of energy and anaerobic conditions that are developed during the storage (Beirão et al., 2019; Rurangwa et al., 2004). The complete preservation and cryopreservation of spermatozoa genomic information should be one of the priorities when a protocol is designed (Cabrita et al., 2010). Sperm cryopreservation can induce DNA fragmentation (single and double strands) in sperm from several marine species. The alterations of DNA can decrease sperm fertility capacity, provoke abnormal development and/or the transmission of heritable damage (Pruski et al., 2009). Therefore, DNA is an important parameter to check after cryopreservation. The Comet assay (single cell gel electrophoresis) revealed that cryopreserved and fresh samples had similar results, tail DNA damage was around 27 to 28%. These values revealed that the level of DNA strand breakage was low, being spermatozoa undamaged. In short, cryopreservation technique used do not seem to provoke damage on *Paracentrotus lividus* spermatozoa DNA. These results are contradictory to some similar studies with other species, where the protection of genetic information is a serious problem (Hossen et al., 2021; Martínez-Páramo et al., 2009).

6. Conclusion

The present study allows to conclude that the provenience of wild purple sea urchin (*P. lividus*) had low influence in sperm quality. Between generations was concluded that captive breeding influence positively sperm quality. The higher sperm motility observed in first captive generation samples and the higher value of viability was in second captive generation. The subpopulation analysis revealed that the *Paracentrotus lividus* spermatozoa can be separated by velocity, and can be used to analyze the degree of maturation. While mean values of motility can be used to immediate observations of this parameter due to its simplicity. Subpopulation analysis can provide more specific information, especially when some procedures will be applied to sperm.

The best sperm to oocyte ratio to obtain the highest percentage of fertilized eggs was 20 000, obtaining 98% fertilized eggs. The results of contact time assay showed that the fertilization took more time to occur than the reported by other authors.

Concerning the comparation between the use of preservation and cryopreservation techniques, it is possible to conclude that for short-term storage sea urchin sperm can withstand quality during six days. However, if there is a need to store sperm for longer times, cryopreservation seems to be advantageous since only a small decrease of viability was registered. In future studies will be necessary to improve the cryopreservation protocol due to some structural damage that affected sperm motility.

7. References

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Appendix 1

Characterization of **Motility Parameters**, given by CASA (VCL, VSL, VAP, LIN STR, WOB, ALH and BCF), of sperm subpopulations (**SP1**, **SP2** and **SP3**) from sperm samples activated with different post activation time (**Time**) and different populations (**Pop.**).

Subpopulations Characterization					
Pop.	Time	Motility Parameters	SP1	SP2	SP3
WN	5 min	VCL ($\mu\text{m/s}$)	106.5 \pm 28.3	35.1 \pm 15.1	
		VSL ($\mu\text{m/s}$)	20.3 \pm 19.6	8.2 \pm 6.7	
		VAP ($\mu\text{m/s}$)	61.6 \pm 24.6	20.1 \pm 8.2	
		LIN (%)	0.2 \pm 0.2	0.3 \pm 0.2	
		STR (%)	0.3 \pm 0.2	0.4 \pm 0.3	
		WOB (%)	0.6 \pm 0.2	0.6 \pm 0.2	
		ALH (μm)	4.8 \pm 1.7	2 \pm 0.8	
		BCF (Hz)	3.2 \pm 3.1	3 \pm 2.1	
	10 min	VCL ($\mu\text{m/s}$)	78.4 \pm 14.6	21.6 \pm 9.6	
		VSL ($\mu\text{m/s}$)	7.2 \pm 6.7	6.4 \pm 5.3	
		VAP ($\mu\text{m/s}$)	35.4 \pm 20.8	13.6 \pm 5.3	
		LIN (%)	0.1 \pm 0.1	0.4 \pm 0.3	
		STR (%)	0.2 \pm 0.2	0.5 \pm 0.3	
		WOB (%)	0.5 \pm 0.2	0.7 \pm 0.2	
		ALH (μm)	3.7 \pm 1	1.5 \pm 0.6	
		BCF (Hz)	2.9 \pm 1.9	2.1 \pm 1.8	
WS/FO	5 min	VCL ($\mu\text{m/s}$)	155.6 \pm 28.3	78.5 \pm 15.9	31.8 \pm 12
		VSL ($\mu\text{m/s}$)	33.3 \pm 32.8	13.8 \pm 13.8	7.1 \pm 6
		VAP ($\mu\text{m/s}$)	115.2 \pm 40.4	41.1 \pm 16.3	18.8 \pm 6.6
		LIN (%)	0.2 \pm 0.2	0.2 \pm 0.2	0.3 \pm 0.2
		STR (%)	0.3 \pm 0.2	0.3 \pm 0.2	0.4 \pm 0.3
		WOB (%)	0.7 \pm 0.2	0.5 \pm 0.2	0.6 \pm 0.2
		ALH (μm)	5.2 \pm 2.5	3.8 \pm 1	1.8 \pm 0.7
		BCF (Hz)	2.7 \pm 2.7	4 \pm 3	3.1 \pm 1.9
	10 min	VCL ($\mu\text{m/s}$)	143.6 \pm 25	88.2 \pm 18.5	29.6 \pm 14.6
		VSL ($\mu\text{m/s}$)	80.6 \pm 35.9	26.1 \pm 18.6	7.5 \pm 5
		VAP ($\mu\text{m/s}$)	134.7 \pm 23.7	59 \pm 22.3	15.9 \pm 7.3
		LIN (%)	0.6 \pm 0.3	0.3 \pm 0.3	0.3 \pm 0.2
		STR (%)	0.6 \pm 0.3	0.4 \pm 0.3	0.5 \pm 0.3
		WOB (%)	0.9 \pm 0	0.7 \pm 0.2	0.6 \pm 0.2
		ALH (μm)	2.4 \pm 0.8	3.4 \pm 1.5	1.7 \pm 0.7
		BCF (Hz)	5 \pm 2.7	3.8 \pm 3	2.9 \pm 2.4

POP.	TIME	MOTILITY PARAMETERS	SP1	SP2	SP3
F1	5 min	VCL ($\mu\text{m/s}$)	156 ± 23.7	52.6 ± 28.5	
		VSL ($\mu\text{m/s}$)	38.7 ± 25.2	18.6 ± 14.6	
		VAP ($\mu\text{m/s}$)	126.6 ± 23	33.1 ± 20.1	
		LIN (%)	0.3 ± 0.2	0.4 ± 0.2	
		STR (%)	0.3 ± 0.2	0.6 ± 0.2	
		WOB (%)	0.8 ± 0.1	0.6 ± 0.2	
		ALH (μm)	4.8 ± 1.4	2.5 ± 1.2	
		BCF (Hz)	2.3 ± 2.3	3.5 ± 2.6	
	10 min	VCL ($\mu\text{m/s}$)	130.6 ± 19.8	43.2 ± 22	
		VSL ($\mu\text{m/s}$)	46.8 ± 32	18.1 ± 13.9	
		VAP ($\mu\text{m/s}$)	112.5 ± 21.5	28.3 ± 16.6	
		LIN (%)	0.4 ± 0.2	0.4 ± 0.2	
		STR (%)	0.4 ± 0.3	0.6 ± 0.3	
		WOB (%)	0.9 ± 0.1	0.7 ± 0.2	
		ALH (μm)	3.3 ± 1.2	2 ± 0.9	
		BCF (Hz)	4.2 ± 3.1	3.6 ± 2.7	
F2	5 min	VCL ($\mu\text{m/s}$)	97.3 ± 26.3	31.6 ± 13.8	
		VSL ($\mu\text{m/s}$)	14.6 ± 13.6	6.8 ± 5.4	
		VAP ($\mu\text{m/s}$)	50.7 ± 28.2	18.2 ± 7.7	
		LIN (%)	0.2 ± 0.2	0.2 ± 0.2	
		STR (%)	0.3 ± 0.2	0.4 ± 0.3	
		WOB (%)	0.5 ± 0.3	0.6 ± 0.2	
		ALH (μm)	4.2 ± 1.5	1.9 ± 0.7	
		BCF (Hz)	3.5 ± 3	3 ± 2.1	
	10 min	VCL ($\mu\text{m/s}$)	104.5 ± 21.4	29.3 ± 15.7	
		VSL ($\mu\text{m/s}$)	25.2 ± 25	8.8 ± 8.7	
		VAP ($\mu\text{m/s}$)	79.9 ± 31.3	19.2 ± 11.3	
		LIN (%)	0.2 ± 0.2	0.3 ± 0.3	
		STR (%)	0.3 ± 0.2	0.5 ± 0.3	
		WOB (%)	0.8 ± 0.2	0.7 ± 0.2	
		ALH (μm)	3.4 ± 1.8	1.7 ± 0.9	
		BCF (Hz)	3.6 ± 2.9	1.9 ± 1.8	